

REMARKS

By Office Action mailed March 7, 2006, pending claims 41-44 stand rejected, reconsideration of which is respectfully requested in view of the above amendments and following remarks. Claim 42 has been amended, claims 41, 43 and 44 have been cancelled and claim 51 has been added. Claims 42 and 51 are now pending.

Rejection Under 35 U.S.C. §101

Claim 44 stands rejected under 35 U.S.C. §101 for the reasons set forth on page 2-3 of the Office Action. In view of Applicants' cancellation of claim 44, Applicants submit that this rejection has been obviated.

Rejections Under 35 U.S.C. §112, First Paragraph

Claims 41-44 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of an enabling disclosure for the reasons set forth on pages 3-9 of the Office Action. More specifically, the Examiner is of the opinion that extrapolating from *in vitro* ICE inhibition to treatment of the autoimmune, inflammatory and neurodegenerative diseases and prevention of ischemic injury generally leads to "unpredictable" results and, therefore, undue experimentation would be required to practice the claimed invention.

Although Applicants disagree with the Examiner's opinion, in order to expedite prosecution, Applicants have (i) amended claim 42 to specify that the inflammatory disease is arthritis, (ii) cancelled claims 41, 43 and 44, and (iii) added claim 51 directed to the treatment of hepatitis. Support for the amendment to claim 42, as well as for new claim 51, may be found at, *e.g.*, page 2, lines 7-16, of the specification. Applicants note that no new matter has been added by way of these amendments. Furthermore, these amendments are not, and should not be construed as, an acquiescence to the Examiner's rejection and Applicants reserve the right to continue prosecution of the cancelled subject matter in one or more related applications. In view of the foregoing, Applicants submit that the pending claims are fully enabled for the following reasons.

With respect to claim 42, as amended, Applicants note that the Examiner indicated that a claim specifically directed to treating arthritis, rather than broadly directed to treating all inflammatory diseases, would be enabled in view of the references cited in Applicants' prior Amendment filed December 19, 2005 (*see* page 6 of the Office Action). Accordingly, Applicants have amended claim 42 in this manner.

With respect to new claim 51, as noted by the Examiner, the claimed compounds have activity as inhibitors of interleukin-1 converting enzyme (ICE) and related proteases, referred to as the ICE/ced-3 family of cysteine proteases. Compounds that inhibit the ICE/ced-3 family of cysteine proteases are useful for a variety of purposes related to the prevention of apoptosis – including the treatment of hepatitis. For example, as demonstrated by Rodriguez et al. (*J. Exp. Med.* 184:2067-2072, 1996 – copy attached), caspase inhibitors (inhibitors of ICE and ICE-like proteases) prevent Fas-mediated fulminant liver destruction and death in mice. As stated by Rodriguez et al. in the "Summary" section (*see* page 2067):

Mice injected with anti-Fas antibody die within a few hours with total liver destruction due to massive apoptosis of hepatocytes. We show that this is preceded and accompanied by the sequential activation of cysteine proteases of the interleukin 1-converting enzyme (ICE) and CPP32 types in the cytosol of the hepatocytes, and that proCCP32 cleavage and enzymatic activity can be prevented by intravenous injections of the tripeptide *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAS.fmk), an inhibitor of ICE-like proteases. ...Injections of the tetrapeptide Ac-YVAD.cmk, more specific for the ICE-like subfamily of cysteine proteases but less cell permeable also gave protection.

Further, as stated in the last paragraph of the Discussion section (*see* page 2071):

The possibility for preventing or attenuating in vivo fulminant liver damage resulting from liver apoptosis has important therapeutic implications in humans. It is generally assumed that fulminant hepatitis occurring during viral hepatitis is mediated by cytotoxic T lymphocytes. The cytotoxic activity of these cells is mediated through the perforin-granzyme and/or the Fas pathway; the mechanisms of cell death are probably common in these two pathways because granzyme B, a serine protease with Asp specificity of cleavage, is an efficient activator of CPP32. The temporary perfusion of peptides with a high cell permeability designed to be potent irreversible inhibitors of the ICE-like proteases may thus be of great therapeutic benefit...

As a further example, as demonstrated by Suzuki (*P.S.E.B.M.* 217:450-454, 1998 – copy attached), a peptide inhibitor specific to the CPP32 subfamily (DEVD-CHO) prevented Fas-mediated liver destruction. As stated by Suzuki in the “Summary” section (*see* page 450):

We propose here that the CPP32 subfamily plays the dominant role in Fas-mediated hepatitis, and DEVD-CHO would be an effective cure for hepatitis.

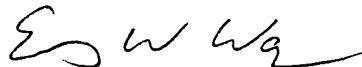
Accordingly, Applicants submit that undue experimentation would not be required to practice the claimed invention, in view of the foregoing (*i.e.*, the teaching of the specification, as further supported by the attached references) and request that this ground of rejection be withdrawn.

In view of the above amendments and remarks, allowance of claims 42 and 51 is respectfully requested. A good faith effort has been made to place this application in condition for allowance. However, should any further issue require attention prior to allowance, the Examiner is requested to contact the undersigned at (206) 622-4900 to resolve the same. Furthermore, the Commissioner is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Respectfully submitted,

Donald S. Karanewsky et al.

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Enclosures:

Rodriguez et al. (*J. Exp. Med.* 184:2067-2072, 1996)

Suzuki (*P.S.E.B.M.* 217:450-454, 1998)

EWV:cw

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Brief Definitive Report**Systemic Injection of a Tripeptide Inhibits the Intracellular Activation of CPP32-like Proteases In Vivo and Fully Protects Mice against Fas-mediated Fulminant Liver Destruction and Death**

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Summary

Mice injected with anti-Fas antibody die within a few hours with total liver destruction due to massive apoptosis of hepatocytes. We show that this is preceded and accompanied by the sequential activation of cysteine proteases of the interleukin 1 β -converting enzyme (ICE) and CPP32 types in the cytosol of the hepatocytes, and that proCPP32 cleavage and enzymatic activity can be prevented by intravenous injections of the tripeptide *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk), an inhibitor of ICE-like proteases. Four Z-VAD.fmk injections at 1-hour intervals abolished all signs of liver damage after anti-Fas antibody injection and resulted in 100% long-range recovery, without residual tissue damage, from a condition otherwise uniformly fatal within <3 hours. This treatment was effective even when delayed until some liver DNA degradation was already detectable. Injections of the tetrapeptide Ac-YVAD.cmk, more specific for the ICE-like subfamily of cysteine proteases but less cell permeable, also gave protection, but at higher doses and when injections started before that of anti-Fas antibody. These observations afford a way of temporarily modulating a number of apoptotic processes in vivo and may have important therapeutic implications in some human diseases.

There is now conclusive evidence that the process of apoptosis or programmed cell death (PCD) results from the activation of members of a new family of cysteine proteases with a specificity of cleavage for aspartate in the P1 position. The decisive importance of this mechanism as an effector of PCD was revealed by the discovery that the *ced-3* gene of *Caenorhabditis elegans*, which is required for cell death occurring during the normal development of this nematode (1), encodes for a protein related to the mammalian IL-1 β -converting enzyme (ICE) (2), an aspartate-specific cysteine protease, and that, in certain conditions, overexpression of ICE itself in mammalian cells can lead to apoptosis (3). Several other members of this protease family have now been identified and are presently subdivided into three subfamilies, each containing variants: the ICE, CPP32 (also called YAMA, apopain, or prICE), and Ich-1 (or Nedd-2) subfamilies; all these enzymes are synthesized as inactive proenzymes requiring cleavage at specific Asp residues to be transformed into active proteases: thus, at least some of these proteases can activate each other in the form of a proteolytic cascade and/or may undergo, once activated, autoprocessing, allowing the potentially lethal ampli-

fication of a minor initial proteolytic process (for review, see reference 4). Short peptides corresponding to the cleavage site of some of these cysteine proteases have been used as inhibitors, with the Tyr-Val-Ala-Asp (YVAD) and the Asp-Glu-Val-Asp (DEVD) motifs being rather specific inhibitors of the ICE and CPP32 subfamilies, respectively (5). Provided their extracellular concentration is high enough, addition of these or related inhibitory peptides to cell cultures prevents many forms, but not all, of PCD (6–8).

One of the best studied ICE-related protease cascades involved in apoptotic cell death is that induced by the triggering of the Fas (APO-1, CD95) receptor on Fas-bearing cells in vitro (6, 7). It has been shown that ICE-like protease(s) and CPP32 are activated in sequence after Fas stimulation (9), and that the first proteolytic event may result from the activation of a novel cysteine protease, which is bound to the FADD or MORT protein (itself bound to the intracellular domain of the Fas receptor) and has a Ced-3-like structure (10, 11). The accumulation of activated CPP32-like proteases thus appears to represent a downstream event in this proteolytic cascade. The most dramatic effect of Fas triggering in vivo as the result of a single injection

tion of an agonistic anti-Fas antibody into mice is the massive apoptotic death of hepatocytes, which results in a few hours in total liver destruction and death (12). In this report we show that this event, which is also accompanied by the sequential activation of ICE- and CPP32-like proteases in the cytoplasm of the hepatocytes, can be completely and permanently prevented by the concomitant injections of high doses of the tripeptide benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk) (13) and a more specific inhibitor of ICE-like proteases, acetyl-Tyr-Val-Ala-Asp chloromethylketone (Ac-YVAD.cmk).

Materials and Methods

Mice. Mice used in this study were 3–4-wk-old (16–18 g) C57BL/6 females bred in the animal facility of the University Medical Center of Geneva.

Anti-Fas Antibody and ICE-like Inhibitor Injections and Histologic Examination. Mice were injected intravenously with 10 μ g of a purified hamster mAb (Jo2) against mouse Fas antigen (12) diluted in 80 μ l of 0.9 g/liter NaCl solution. Z-VAD.fmk (Enzyme Systems Products, Dublin, CA), Z-DEVD.cmk, and Ac-YVAD.cmk peptides (BACHEM Feinchemikalien AG, CH), were dissolved in DMSO at a concentration of 100 mg/ml. All peptide injections were intravenous and started 5 min after anti-Fas antibody injection, except when indicated otherwise. Mice were killed at various times or autopsied immediately after death, and fragments of tissues were fixed *in vivo* with 4% paraformaldehyde in PBS, embedded in paraffin, and 5- μ m sections were stained with hematoxylin and eosin.

Western Blot Analysis. Minced liver fragments were homogenized at 4°C with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 2 μ g/ μ l aprotinin and 1 mM PMSF. After centrifugation at 15,000 g, 30 μ g of supernatant protein was loaded per lane on 10% polyacrylamide gels and electrophoresed. After transfer to nitrocellulose membranes and exposure to 5% nonfat milk TBS-T (20 mM Tris HCl, 500 mM NaCl, pH 7.5, and Tween 0.1%), the washed membranes were incubated for 1 h at 20°C with a rabbit anti-murine ICE at a 1:3,000 dilution (kind gift of J. Tschoopp, Institut de Biochimie, ISREC, Lausanne, Switzerland), a rabbit anti-ICE p10 at a dilution of 1:200 (M20; Santa Cruz Biotech Inc., CA), a rabbit anti-human CPP32 p17 at a dilution of 1:7,500 (kind gift of D. Nicholson, Merck Frosst, Pointe-Claire-Dorval, Quebec, Canada), or anti-LAP3 (kind gift of V.M. Dixit, University of Michigan, Ann Arbor, MI). Goat anti-rabbit IgG (Santa Cruz Biotech, Inc., Santa Cruz, CA) was used as second antibody at a concentration of 400–800 ng/ml. Membranes were washed with TBS-T, incubated in enhanced chemiluminescence detection reagents (Amersham International, Amersham, UK) at room temperature, and exposed to Hyperfilm-MP films (Amersham International).

Fluorometric Analyses. Liver protein extracts were prepared by Dounce homogenization of 20 mg of tissue in a hypotonic buffer (25 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 1 μ g/ml leupeptin and aprotinin). Homogenates were centrifuged at 15,000 rpm for 10 min and the supernatants were used. 20 μ g of the extracted proteins was incubated with the fluorescent substrates Z-YVAD-AFC or Z-DEVD-AFC (Enzyme Systems Products) at a concentration of 25 mM in 50 mM Hepes, 1% sucrose, 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate), and 5 mM dithiothreitol in a volume of 1 ml. The fluorescence of the cleaved substrates was

determined using a spectrofluorometer set at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. One unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 25°C in 30 min.

DNA Fragmentation Detection. For the detection of oligonucleosomes, a cell death detection ELISA kit (Boehringer Mannheim AG, Rotkreuz, Switzerland) was used according to the manufacturer's instructions adapted for the use of tissue fragments. Briefly, small pieces of liver (25–40 mg) were weighed and homogenized in the provided lysis buffer, incubated for 30 min at room temperature, and after a 10-min centrifugation at 2,000 rpm, 20 μ l of the supernatant was submitted to the ELISA test. For electrophoretic detection of DNA cleavage products, liver extract supernatants were precipitated overnight with isopropanol. The 15,000-rpm pellets were then digested overnight with proteinase K, extracted with phenol-chloroform-isoamylalcohol, treated with RNase A, precipitated with ethanol, and submitted to electrophoresis in 2% agarose gels.

Serum Aminotransferase Determination. Quantification of alanine aminotransferase (ALT) and aspartate aminotransferase (ASAT) in the sera was made with a standard clinical automatic analyzer (model 7150; Hitachi).

Results

Intravenous injection of 10 μ g of the Jo2 anti-Fas mAb was used throughout these experiments. This amount invariably killed all 15–18-g C57BL/6 mice within 3 h; when killed after 2 h, all mice already had a severely shrunken, massively hemorrhagic liver, with extensive lesions of hepatocyte apoptosis on histologic sections (Fig. 1 *b*, compared with normal liver, Fig. 1 *a*). Evidence of hepatocyte damage became clearly detectable only after 60 min; at this time, blood levels of liver aminotransferases were almost unchanged (Fig. 2 *a*), and in liver lysates DNA oligonucleosomes characteristic of apoptosis were only detectable by a sensitive immunologic assay (Fig. 2 *b*); after 60 min, the blood aminotransferases rapidly increased to very high levels until death (Fig. 2 *a*) and DNA fragmentation in the liver, as detected on agarose gels, became massive (Fig. 2 *b*). Proteolytic activity of the ICE and CPP32 type was as-

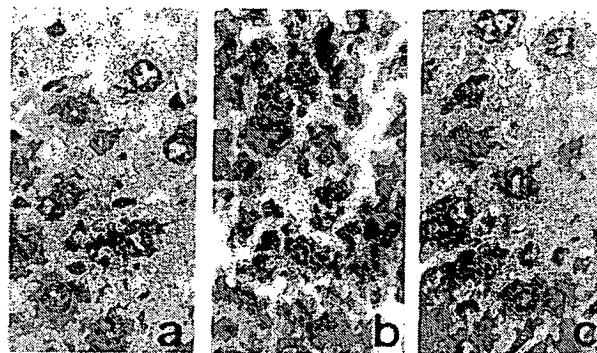


Figure 1. Liver histology, detail. (*a*) Normal mouse. (*b*) Mouse killed 2 h after injection of anti-Fas antibody. The architecture of the liver parenchyma is destroyed with hemorrhagic foci, several apoptotic hepatocytes are seen. (*c*) Mouse killed 2 h after antibody injection, followed after 5 min by injection of 500 μ g Z-VAD.fmk. Original magnification $\times 400$.

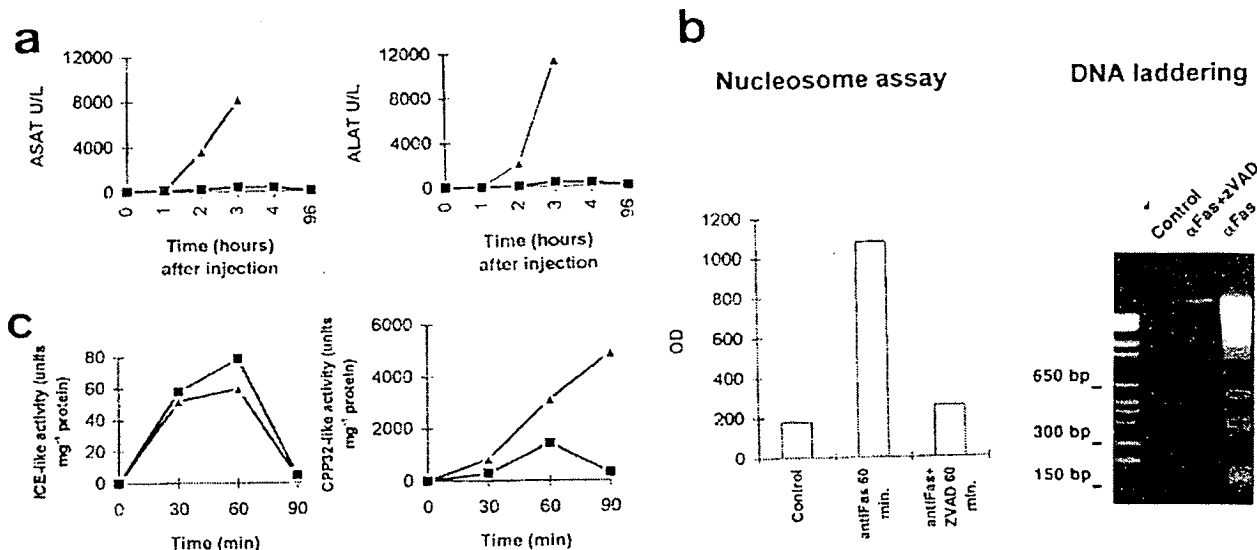


Figure 2. (a) Prevention by Z-VAD.fmk injections of the increase of ASAT (left) and ALAT (right) concentrations in the serum after Fas-induced liver damage. \blacktriangle , control mice; \blacksquare , treated mice. (b) Prevention of Fas-induced liver DNA fragmentation after Z-VAD.fmk injections. (Left) DNA fragmentation assay (nucleosome). Normal liver (left column); middle: liver 1 h after anti-Fas antibody injection (middle column); effect of Z-VAD.fmk injection 5 min after anti-Fas injection (right column). (Right) DNA laddering on agarose gel 2 h after anti-Fas injections, with or without Z-VAD.fmk injection. (c) Activation of ICE-like and CPP32-like proteases during Fas-induced liver damage in vivo, using cleavage assays of the fluorescent substrates Z-YVAD-AFC (for ICE-like proteases; left) and Z-DEVD-AFC (for CPP32-like proteases; right) in the presence of the same liver cytosolic extracts used for both assays. \blacktriangle , control; \blacksquare , Z-VAD-treated mice.

sessed in the liver lysates of mice killed at 30-min intervals after antibody injection, by using as substrates the Z-YVAD-AFC and Z-DEVD-AFC peptides, which correspond, respectively, to an ICE and a CPP32 cleavage site (5). ICE-like activity rapidly rose in liver lysates after 30 min but in a transient way, and returned to normal values at 90 min; in contrast, CPP32-like activity rose continuously until 120 min, at the time of very severe hepatocyte damage (Fig. 2 c). Cleavage of proICE and proCPP32 was also directly explored by Western blotting of the liver lysates (Fig. 3). A very limited cleavage of proICE, indicated by the appearance of a 36-kD band (14), was detectable after 60 min; a decrease in proCPP32 was observed after 60 min and corresponded to a limited cleavage of the proenzyme as shown by the detection of a 17-kD band of weak intensity. No LAP-3, a protease of the CPP32 family, was detectable, suggesting that this enzyme is not present in the liver (15). The nuclear poly(ADP-ribose) polymerase, a substrate for CPP32 whose cleavage is characteristic of apoptotic nuclei (16), could not be clearly visualized, perhaps because this protein is less abundant in the nuclei of the nondividing hepatocytes than in those of dividing cells, where it is usually observed.

Mice were then injected with anti-Fas antibody followed 5 min later by intravenous injections of various amounts of the Z-VAD.fmk peptide. In a pilot experiment, two mice injected with 1 mg or 0.25 mg (about 2 or 0.5 μ M, respectively) of the peptide survived 15 and 7 h, respectively, but had severely damaged liver at autopsy, compara-

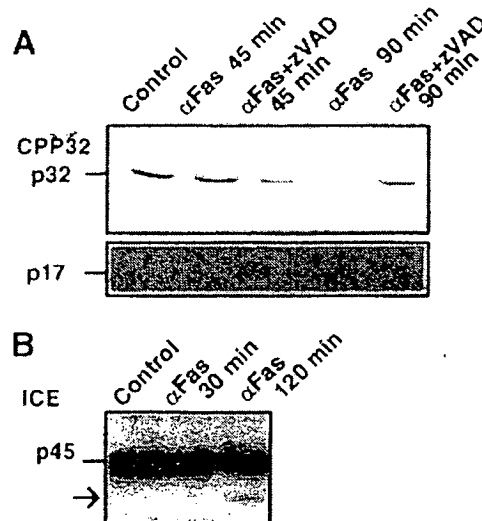


Figure 3. Western blots for the detection of CPP32 and ICE in liver cytosolic extracts. (a) Anti-CPP32 antibody. In addition to the p32 band of proCPP32, a band of p17 is present in the liver 90 min after antibody injection and is not detectable in the liver lysates from a Z-VAD.fmk-treated mouse. Exposure time of the film was 5 min for p45 and 15 min for p17. (b) Anti-ICE antibody: a faint band (p36, arrow), corresponding to the initial processing of proICE (p45) is distinguishable in liver lysates after anti-Fas antibody injection. Whereas in other experiments the faint p36 band was seen after 60 min, here it was more clearly seen in the 120-min liver lysate.

ble to that of control mice, which died within 3 h; three mice injected with 0.5 mg of the peptide were killed after 2 h and had macroscopically and histologically normal liver (Fig. 1 c). On this basis, it was decided to prolong exposure to the protecting peptide by using the following schedule: an initial injection of 0.25 mg, followed by three injections of 0.1 mg at 1-h intervals. Three experiments were then performed, corresponding to a total of 15 mice injected according to this schedule and 20 control mice: all control mice died within 3 h, whereas the 15 Z-VAD-injected mice survived and were in apparent good health days or weeks later. In a last experiment involving five mice and their controls, this peptide injection schedule was delayed until 65 min after anti-Fas antibody injection, i.e., at a time when apoptotic DNA fragmentation in the liver had already started (Fig. 2 b). Four out of the five Z-VAD-injected mice survived and one died after 10 h with a severely damaged liver.

Mice protected by this injection schedule started 5 min after antibody injection were then killed or bled at different times to follow the cysteine protease activity of their liver and their blood level of aminotransferases. Unexpectedly, the first wave of Z-YVAD-AFP cleavage activity of the hepatocyte cytosol was not decreased (Fig. 2 a, left); in contrast, the Z-DEVD-AFP cleavage activity was decreased and had returned to baseline levels after 90 min, at a time when unprotected mice showed a very high activity (Fig. 2 a, right). In agreement with this last observation, no cleavage of CPP32 was detected on Western blots (Fig. 3). Blood aminotransferase levels showed only a very small increase compared with that of unprotected mice and were at the baseline levels 4 d later (Fig. 2 a). Histologic examination of the liver obtained at various times of sacrifice 1 to several days later showed no signs of damage, indicating full protection. In contrast, one of the four mice surviving after the 65-min-delayed Z-VAD injection schedule mentioned above and sacrificed 1 d later showed histologically focal areas of liver necrosis; these lesions can certainly heal, because the three other surviving mice of this group were in apparent good health in the following weeks. The histologic appearance of various organs (heart, spleen, gut, lung, brain, intestine) was also normal, suggesting that the injected peptides, at the dosage used, had no toxic effect. It should be added that protection of the liver in all these experiments cannot be attributed to an unexpected effect of a Z-xmk compound on hepatocytes, since mice injected with the same amount of the Z-D.cmk compound died, as did control mice, with identical lesions.

A more specific inhibitor of cysteine proteases of the ICE subfamily is the tetrapeptide Ac-YVAD.cmk. In cell culture, it is, however, more difficult to inhibit Fas-mediated apoptosis with this peptide. Using mouse Fas-transfected W4 and P815 cell lines, we have observed that Fas-mediated apoptosis, which is otherwise almost complete within 4 h, is highly inhibited at concentration of 100 μ M by Z-VAD.fmk, but only partially inhibited in the presence of 300–600 μ M Ac-YVAD.fmk, perhaps because of the

lesser cell permeability of the latter compound. Two mice were injected with Ac-YVAD.cmk according to the schedule described above, but with a double amount (i.e., initial dose of 0.5 mg): one mouse died after 7.5 h and the other survived (but its liver was not examined). Because the intracellular availability of the peptide may be slower, the peptide administration schedule was applied to one mouse starting 15 min before anti-Fas injection; this mouse survived and had no gross or histologic liver lesions when sacrificed 1 d later. In spite of their small number, these last results conclusively show that administration of a peptide of more restricted anti-ICE specificity is capable of preventing Fas-mediated apoptosis *in vivo*.

Discussion

The cysteine protease activity triggered in hepatocytes *in vivo* by anti-Fas antibody injections showed a pattern of sequential activity comparable to that observed during Fas-induced apoptosis of mouse W4 cells (9), with a transient ICE-like activity observed first, followed by a gradually increasing CPP32-like activity. However, whereas on Western blots some degree of cleavage of proICE was observed after 45 min and of proCPP32 after 90 min, these cleavages were much weaker than those observed on Western blots of W4 and P815 cells undergoing *in vitro* Fas-induced apoptosis (Ody, C., K. Matsuura, and P. Vassalli, unpublished observation). This observation suggests that other members of the Ced-3 cysteine proteases family may be more preferentially involved in hepatocyte PCD; the liver appears to be especially rich in ICE_{rel} II and III proteases (belonging to the ICE subfamily) and in Mch2 (considered to belong to the CPP32 subfamily), the cleavage of which could not be explored because of the lack of relevant antibodies. Some of these last proteases appear to be more susceptible to some protease inhibitors than other members of their subfamily (17). This may explain why hepatocytes may be protected by the Z-VAD and YVAD inhibitory peptides more easily than W4 or P815 cells, because it is not likely that the concentration of these peptides required for *in vitro* prevention of Fas-mediated apoptosis of these cell lines, as described above, had been durably achieved *in vivo*. The observation that ICE-like protease activity was not inhibited in the liver of the Z-VAD protected mice, in contrast to CPP32-like activity, might suggest that Z-VAD.fmk is a better inhibitor of the latter enzymes. However, because it has been observed that Z-VAD.fmk inhibits poorly *in vitro* CPP32-like proteolysis once activated (18), it is more likely that the failure of Z-VAD injections, with the injection schedule used, to efficiently inhibit the early ICE-like protease activity, in contrast to the late CPP32-like activity, results from an insufficiently high intracellular concentration of the inhibitory peptides after the first injection. The observation that injections of the Ac-YVAD.cmk peptide also completely prevented liver damage when started before anti-Fas antibody injection strongly supports the notion of a PCD-inducing proteolytic cascade that can be interrupted

at its early stages. However, the first element of the cascade in Fas-induced PCD is the activation of the MACH-FLICE cysteine protease, bound to the MORT(FADD)-Fas protein complex (10, 11). This protease, which belongs to the CPP32 subfamily, cleaves after its activation the Z-DEVD- but not the Z-YVAD-AFC peptide (10). Thus, the early autoamplification steps of the cysteine protease cascade, rather than the probable initial proteolytic event, appear to be the targets of the inhibitory peptides used to prevent hepatocyte death in these experiments.

An intriguing question raised by the obvious instability and rapid decrease *in vivo* of the inhibitory peptides is how full and permanent protection against liver damage and death can be achieved by a few hours of protection after injection of an amount as high as 10 µg of agonist antibody, which is not expected to diffuse very rapidly out of the vascular compartment. At least three not mutually exclusive possibilities must be considered, which are presently under study: rapid removal of the antibody by Fas molecules makes it drop to a level insufficient to trigger significant hepatocyte damage; the level of Fas molecules on hepatocytes may also drop and lead to the same result; protected hepatocytes may undergo metabolic changes making them more resistant to Fas-mediated death, as occurs with TNF-sensitive cells first exposed to a sublethal concentration of TNF (19).

The possibility of preventing or attenuating *in vivo* fulminant liver damage resulting from liver apoptosis has important therapeutic implications in humans. It is generally assumed that fulminant hepatitis occurring during viral

hepatitis is mediated by cytotoxic T lymphocytes. The cytotoxic activity of these cells is mediated through the perforin-granzyme and/or the Fas pathway (20); the mechanisms of cell death are probably common in these two pathways because granzyme B, a serine protease with Asp specificity of cleavage, is an efficient activator of CPP32 (21–23). The temporary perfusion of peptides with a high cell permeability designed to be potent irreversible inhibitors of the ICE-like proteases (24, 25) may thus be of great therapeutic benefit if these compounds show no general toxicity. In the very limited pattern of injection used in the present experiments, no signs of toxicity were observed; Z-VAD.fmk *in vitro* at a concentration of 100 µM has been found not to be toxic for at least 48 h (26). Our observation that Z-VAD.fmk injections protected mice from massive liver destruction and death even when delayed until a time when apoptotic DNA cleavage was detectable strengthens the potential therapeutic value of this approach. A number of other acute clinical situations are probably accompanied by cell death, especially harmful in tissues where, in contrast to hepatocytes, regeneration does not occur, such as the nervous tissue; *in vitro*, Z-VAD.fmk has been found to protect neurons from certain apoptotic-inducing conditions (27). Finally, perfusion of cysteine protease inhibitory peptides may be also of experimental use to detect *in vivo* the involvement of PCD in physiological or pathological conditions, as well as to explore the mechanism underlying various PCD, because *in vitro* not all PCD-inducing conditions display sensitivity to inhibitory peptides such as Z-VAD.fmk.

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The Dominant Role of CPP32 Subfamily in Fas-Mediated Hepatitis (44256)

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Abstract. Fas is a cell surface molecule that transduces the apoptotic death signaling on the stimulation of Fas ligand, and plays the dominant role in various disease states. The lethal effect of Fas antibody in mice has been reported, and this experimental procedure has been used as the model for hepatitis. Recently, the prevention of this Fas antibody-induced hepatitis by the broad caspase inhibitor (z-VAD.fmk) has been reported. In the present study, we additionally demonstrated that the CPP32 subfamily, rather than the ICE subfamily, plays the dominant role in the Fas antibody-induced hepatitis. Fas antibody-injection induced chromosomal DNA fragmentation and CPP32 subfamily-activation in both the liver and lung. Tissue damage observed in the lung was weak as compared with liver damage. When mice were exposed to DEVD-CHO (specific inhibitor of CPP32 subfamily), this lethal effect of Fas antibody, tissue destruction, and CPP32 subfamily-activation were prevented. In contrast, YVAD-CHO (specific inhibitor of ICE subfamily) could not prevent the lethal effect of Fas antibody. We propose here that the CPP32 subfamily plays the dominant role in Fas-mediated hepatitis, and DEVD-CHO would be an effective cure for hepatitis.

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Cell surface molecule Fas, a type-I transmembrane protein belonging to the nerve growth factor/tumor necrosis factor receptor family, transduces the apoptotic death signaling on stimulation with Fas ligand or anti-Fas antibody (Fas Ab) (1-3). Fas plays the dominant role in physiological cell death, and its endogenous expression is encountered in liver, lung, thymus, heart, ovary, and vagina (4, 5). Recently, the lethal effect of Fas Ab in mice was reported, in which it was shown that this lethal effect was due to fulminant liver destruction (6). Various investigations into the molecular machinery of various types of hepatitis have also demonstrated the direct involvement of Fas ligand/Fas system (7-9), and Fas Ab injection into mice was established as the model for hepatitis (10, 11).

CED-3 death gene, identified from *Caenorhabditis elegans*, shows high similarity to interleukin-1 β converting enzyme (ICE) (12, 13). Recently, various ICE/CED-3 ho-

mologs that were recently termed "caspase (14)" have been identified, and an important role in apoptotic cell death has been reported. Especially, CPP32 subfamily (15) plays the dominant role in apoptotic death signaling (16-19). On the basis of the machinery by which they induce apoptotic death signaling, two types of CPP32 subfamily are known: one is CPP32/Yama/Apopain (caspase 3), which does not physically interact with the cytoplasmic region of Fas; and another is FLICE/MACH (caspase 8), which can directly interact with the death domain of Fas (18, 19). In Fas-mediated apoptosis, caspase 3 is activated by ICE subfamily-activation (ICE cascade; Ref. (16)). Thus, the CPP32 subfamily plays the dominant role in the downstream of Fas-initiated death signaling.

Bcl-2 oncoprotein was originally identified through study of the t(14; 18) translocation present in human B-cell follicular lymphomas (20), and is unique in that it inhibits apoptosis rather than promoting cell proliferation (21, 22). Bcl-2 prevents Fas-mediated apoptosis *in vitro* (23). Because Bcl-2 expression is not encountered in liver (24), the effect of Bcl-2 in Fas Ab-induced fulminant liver destruction was investigated in a Bcl-2 transgenic mouse. Overexpression of Bcl-2 in mouse liver showed the resistance to Fas Ab-induced fulminant liver destruction (10, 11). It is thought that Bcl-2 prevents the upstream portion of the ICE cascade (25). Therefore, this finding in Bcl-2 transgenic

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mice suggests that the prevention of Fas-initiated death signaling would be an effective cure for hepatitis. In addition, the prevention of Fas Ab-induced hepatitis by the systemic injection of broad caspase inhibitor to mice has recently been reported (26). In the present study, therefore, we examined the protective effect of the CPP32 subfamily specific peptide-inhibitor DEVD-CHO in Fas Ab-induced lethal effect.

Materials and Methods

Fas Ab- or DEVD-CHO-injection. Male Slc/ddy, 5-week-old mice were used for experiments. Sixteen mice were used in each group. Anti-mouse Fas antibody (Jo2 clone and Ref. 6) was purchased from Pharmingen (San Diego, CA) and diluted with PBS. Ten micrograms of Fas Ab were injected intravenously, with an equal amount of hamster IgG diluted with PBS injected as control. After injection, lung, heart, liver, and thymus were removed, and used for chromosomal DNA analysis, histological analysis, and the measurement of CPP32 subfamily-activity. CPP32 subfamily-inhibitor DEVD-CHO was purchased from Peptide Laboratories (Osaka, Japan) and dissolved with DMSO. The DMSO-dissolved DEVD-CHO was diluted from PBS (final conc. of DMSO is 1%), and 0.1 or 0.5 mg of DEVD-CHO were injected intravenously. Each experiment was performed three times.

Chromosomal DNA Analysis. To examine whether Fas Ab injection induces apoptosis in each tissue, chromosomal DNA fragmentation, one of apoptotic fashion (27), was analyzed as previously described (5, 28). Tissues were digested with lysis buffer 100 mM NaCl, 25 mM ethylenediaminetetra-acetic acid (EDTA) (Wako), 100 mM Tris-HCl (pH 8.0), 0.5% SDS, and 0.3 mg/ml proteinase K (Wako) at 50°C for 14 hr after homogenization. Samples were extracted three times with phenol/chloroform/isoamyl alcohol (PCIA; 25:24:1) and then once with chloroform. An equal volume of isopropanol was added to the aqueous phase, and then DNA was precipitated for 1 hr at -20°C. The DNA was dissolved in 10 mM Tris 50 mM EDTA (pH 8.0), and then treated with 50 µg RNase (Wako) for 1 hr at 37°C. After final PCIA extraction, DNA was precipitated as described above. Purified DNA was measured spectrophotometrically at A260/A280, and the same amount of DNA (1 µg/lane) was electrophoretically separated in 2% agarose gel in TBE (45 mM Tris/45 mM boric acid/1 mM EDTA) for 1 hr at 100 volts/hr. Gels were stained with ethidium bromide (0.5 µg/ml) for 5 min and rinsed with distilled water.

Histological Analysis. Tissues from mice were fixed in formalin, embedded in paraffin, and serially sectioned at 8 µm. The sections were stained with Delafield's hematoxylin and eosin (HE) or Hoechst 33342. The Hoechst 33342 staining procedure was performed as previously described (17) with some modifications. Sections were treated with xylene and EtOH, and then washed with PBS. After washing, sections were reacted with Hoechst 33342 (1 µM) for 18 hr.

Protein Extraction and Measurement of CPP32 Subfamily-Activity. Protein extraction and the enzyme assay were performed as previously described (17) with some modifications. Tissues were removed, washed with PBS, and minced in PBS containing 1 mM EDTA. After the addition of 10 µM (final conc.) digitonin (Sigma Chemical Co., St. Louis, MO), tissues were incubated at 37°C for 30 min. Lysates were collected by centrifugation (15000 rpm/5 min), and protein concentration was measured using a DC protein assay kit (Bio Rad, Hercules, CA).

For assay of CPP32 subfamily-activity, aliquots were incubated with 10 µl of DEVD-MCA (Peptide Lab.; 50 µM), and the release of amino-4-methylcoumarin was monitored with a spectrofluorometer.

Results and Discussion

When mice (Slc/ddy; male, 5 weeks old) were injected intravenously with 10 µg of anti-mouse Fas antibody (Fas Ab; Jo2 clone and Ref. 6), all Fas Ab-injected mice died within 6 hr (Fig. 1). To examine tissue damage, chromosomal DNA analysis and histological study were performed in Fas-expressing tissues, namely lung, heart, liver, and thymus (4). In control mice (hamster IgG-injected mice and Ref. 6), tissue damage was not encountered in all tissues (Fig. 2). In contrast, tissue damage detected by chromosomal DNA fragmentation was detected in lung, liver, and thymus of Fas Ab-injected mice, but not in heart (Fig. 2a). It has been reported that intraperitoneal injection of Fas Ab induces thymocyte apoptosis, and that CD4⁺/CD8⁺ thymocytes die as the target of Fas Ab (29). We suggest that the apoptosis encountered in thymus of Fas Ab-injected mice is due to CD4⁺/CD8⁺ thymocyte apoptosis. HE- and Hoechst 33342-staining analysis revealed that cells in the liver and

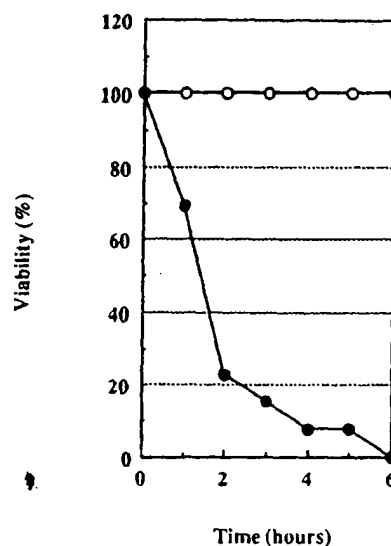


Figure 1. Lethal effect of Fas Ab in mice. 100 µl of 1% DMSO in PBS (DMSO-PBS) was injected intravenously, followed by intravenous injection of Fas Ab 10 µg (closed circle) or hamster IgG (open circle).

a

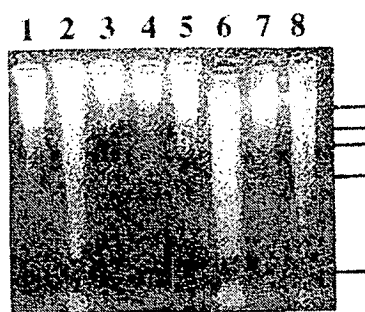
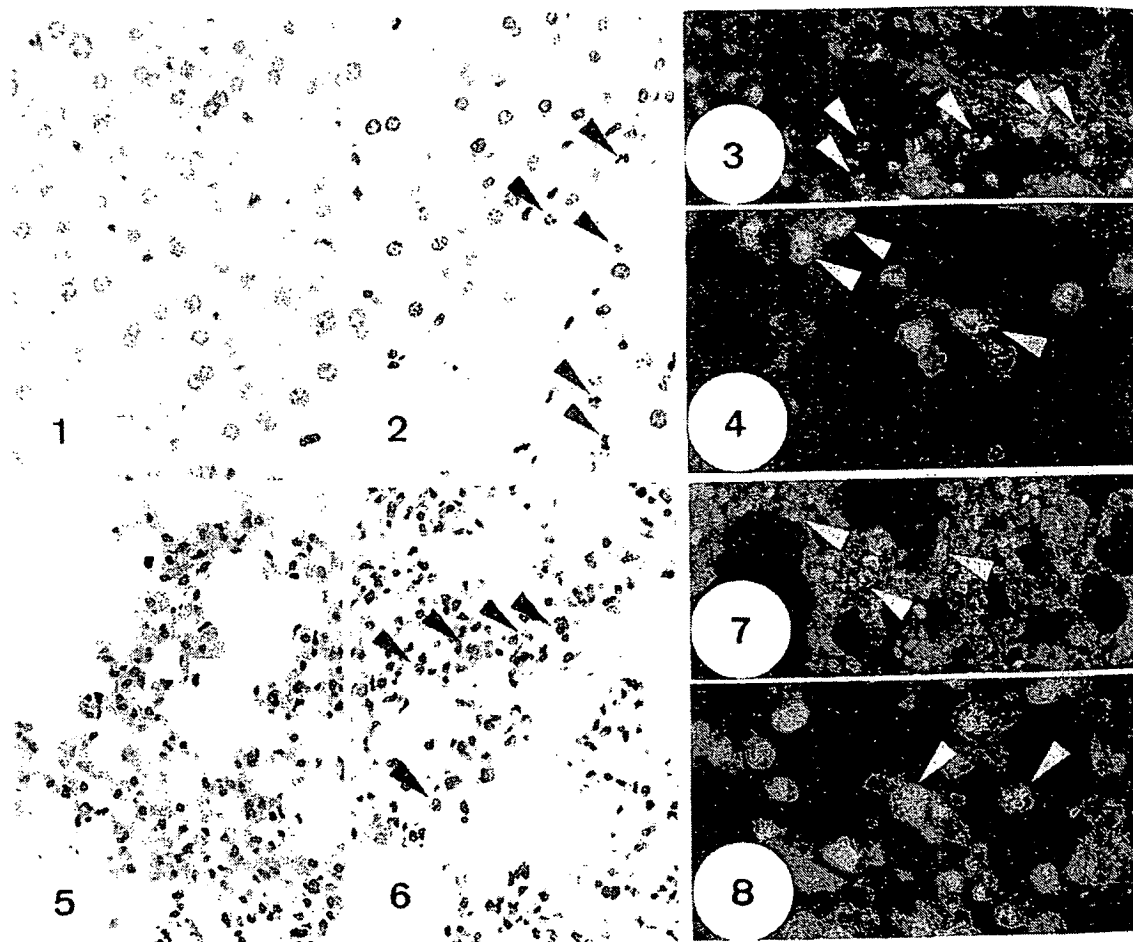


Figure 2. Tissue damage induced by Fas Ab-injection. (a) Chromosomal DNA fragmentation analysis. Chromosomal DNAs were extracted from lung (Lanes 1 and 2), heart (Lanes 3 and 4), liver (Lanes 5 and 6) or thymus (Lanes 7 and 8) of mice injected with hamster IgG (Lanes 1, 3, 5, and 7) or Fas Ab (Lanes 2, 4, 6, and 8). Chromosomal DNAs extracted from tissues of Fas Ab-injected mice that died at 2 hr after injection were indicated. Chromosomal DNAs were separated on 2% agarose gels and stained with EtBr. Bars on the right of the photograph show the position of DNA marker 1357, 1078, 872, 603 and 194 bp (from top to bottom). (b) Histological analysis of liver and lung damage. Liver (upper panels: 1-4) and lung (lower panels: 5-8) were removed from intact mice and stained with HE (1 and 5; 400 \times). Liver and lung of Fas Ab-injected mice that died at 2 hr after injection were also removed and stained with HE (2 and 6; 400 \times) or Hoechst 33342 (3, 4, 7, and 8; 3 and 7: 400 \times ; 4 and 8: 1000 \times). Arrowheads (black and yellow in each panel) show the typical apoptotic cells showing nuclear condensation and/or fragmentation.

b



lung showed some typical apoptotic fashion (27), such as nuclear fragmentation and condensation (Fig. 2b), whereas lung damage was very weak as compared with liver damage. We suggest that the lethal effect of Fas Ab is dominantly due to fulminant liver destruction, and that light lung damage may cause some effect to the lethal effect of Fas Ab.

Hasegawa *et al.* reported the dominant role of the

CPP32 subfamily in Fas-mediated apoptosis (17). In addition, if the ICE cascade operates in Fas-mediated hepatitis, the CPP32 subfamily plays the dominant role at the downstream portion (16, 30, 31). Therefore, the direct involvement of the CPP32 subfamily in Fas Ab-induced tissue damage was examined. Because the CPP32 subfamily cleaves poly (ADP-ribose) polymerase (PARP) (15, 32), MCA-modified tetrapeptide (DEVD-MCA) coding the

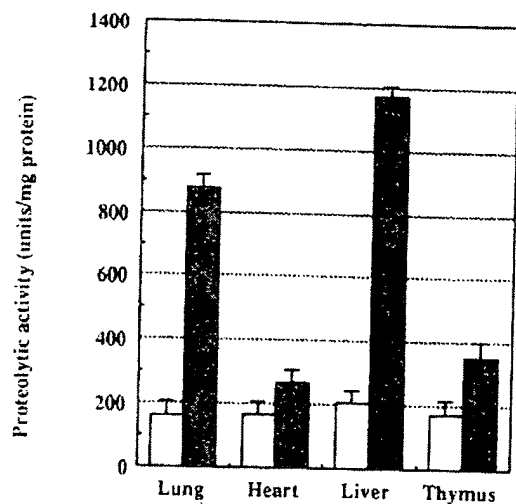


Figure 3. Proteolytic activity of CPP32 subfamily. Proteins were extracted from tissues from mice injected with hamster IgG (open column) or Fas Ab (closed column).

cleavage site of PARP can be used as the substrate (17). When mice were exposed to Fas Ab, a drastic elevation of CPP32 subfamily-activity was encountered in lung and liver, but not in heart and thymus (Fig. 3).

Various investigations have reported that CPP32 subfamily-activation initiated by Fas is prevented by the synthesized tetrapeptide inhibitor DEVD-CHO *in vitro* (17, 32, 33). Therefore, we examined the effect of DEVD-CHO on the lethal effect of Fas Ab. Mice were intravenously preinjected with DEVD-CHO, and then exposed to Fas Ab. Even single injection of DEVD-CHO rescued 18.75% (0.1 mg/mouse), 50% (0.5 mg/mouse) 75% (1 mg/mouse) or 100% (5 mg/mouse) of the mice from the lethal effect of Fas Ab

(Fig. 4a), and fulminant liver destruction was also prevented (Fig. 4b). Broad caspase inhibitor z-VAD.fmk also rescued 100% of the mice from the lethal effect of Fas Ab by the 1-hr interval injection after Fas Ab-injection (26). In the present study, single injection of DEVD-CHO (5 mg/mouse) was effective (100% rescue) even 30 min after Fas Ab-injection (data not shown). The effect of a single injection of ICE inhibitor (YVAD-CHO) on the lethal effect of Fas Ab was also examined. Whereas, YVAD-CHO injection (0.5 mg/mouse) could rescue only 33.3% of the mice from the lethal effect of Fas Ab, and 15 mg/mouse were required to rescue 100% of the mice from the lethal effect of Fas Ab (data not shown). Therefore, we propose here that DEVD-CHO is more effective than other caspase inhibitors. In addition, DEVD-CHO exposure to mice completely prevented Fas Ab-induced CPP32 subfamily-activation (Fig. 4c).

The present study demonstrated that Fas Ab-injection in mice induced liver and lung damage mediated by CPP32 subfamily activation. In general, an abnormality of the lungs in patients carrying hepatitis has been known. Although, the dominant role of the Fas ligand/Fas system in hepatitis has already been demonstrated, there is no report that suggests the possible involvement of the Fas ligand/Fas system in lung damage. The lethal effect of Fas Ab was prevented by the pre- and postinjection of DEVD-CHO. The administration of DEVD-CHO may be effective in the treatment of hepatitis. Various disease states triggered by caspase activation have been reported (3, 34). In addition, chemotherapeutic agents also induced apoptosis accompanied by caspase activation (31, 35, 36) and showed various side effects. The current results additionally suggest that DEVD-CHO may become the basic model of the new drug that is

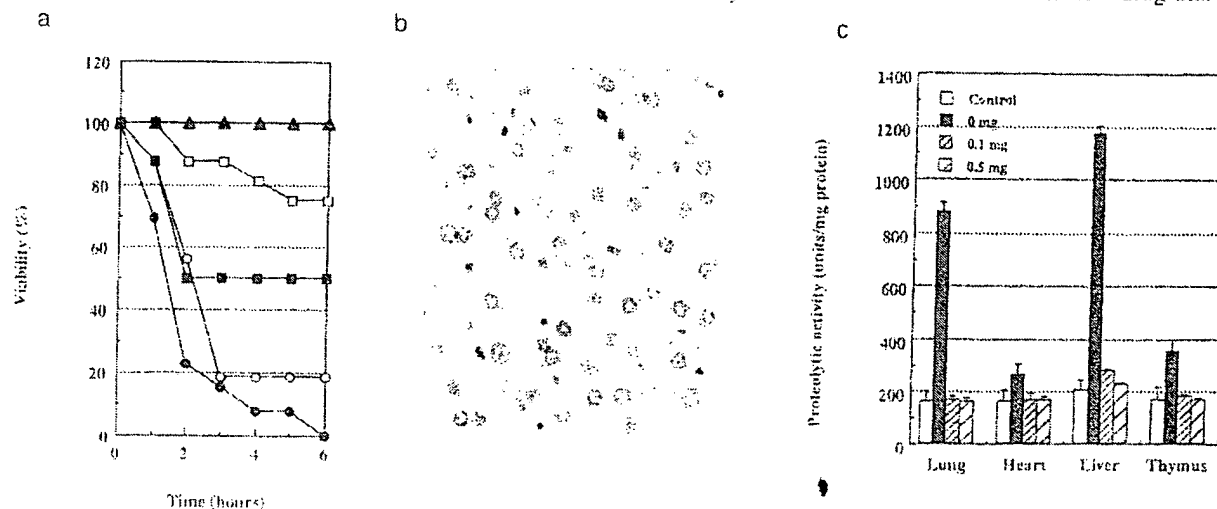


Figure 4. Effect of DEVD-CHO on Fas Ab-induced lethal effect and fulminant liver destruction. (a) Resistance to the lethal effect of Fas Ab in mice. Mice were injected intravenously with 100 μ l of DMSO-PBS (closed circle), or 0.1 (open circle), 0.5 (closed square), 1 (open square) or 5 (closed triangle) mg of DEVD-CHO. After 2 hr, mice were injected intravenously with 10 μ g of Fas Ab. (b) Histological analysis of liver from DEVD-CHO pretreated mice. Liver was removed from both DEVD-CHO (0.5 mg) and Fas Ab-treated mice that were alive at 6 hr after Fas-Ab injection and stained with HE (400x). As compared with intact mice liver (Fig. 2b-1), there was no significant difference. (c) Effect of DEVD-CHO on proteolytic activity of CPP32 subfamily. Proteins were extracted from tissues of rescued mice. Mice injected with DMSO-PBS (0 mg), or 0.1 (0.1 mg) or 0.5 (0.5 mg) mg of DEVD-CHO. After 2 hr, mice were injected intravenously with 10 μ g of Fas Ab.

an effective cure of cell death-associated diseases and side effects.

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